Investigations into Endophytes in *Pimenta dioica* (L.) Merr.

Submitted By:

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Synopsis of the proposed work for the award of degree

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INTRODUCTION

The term ‘endophyte’ was coined by de Bary in 1866 to refer microbes that inhabit plant tissues at some stages in their life cycle, without causing apparent harm to their host. All vascular plants examined till date have been reported to harbour endophytic microorganisms, which commonly are bacteria and fungi (Zhang et al. 2006). Endophytes are capable to colonize leaves, petioles,stems, twigs, bark, root, fruit, flower and seeds. In general endophytes enter plant through root, flower, leaf, stem, and cotyledons (Kobayashiand Palumbo 2000), and they may either become localized at the point of entry or spread throughout the plant. In accordance with their life strategies, endophytes can be either ‘obligate’ or ‘facultative’. Obligate endophytes are strictly dependent on the host plant for their growth and survival, and their transmission to other plants occurs vertically or via vectors (Su et al. 2010). Facultative endophytes have a stage in their life cycle in which they exist outside host plants (Rajkumar et al. 2009; Su et al. 2010).

Endophytes are ubiquitous, beneficial microbes that help in various aspects of plant growth and development such as nitrogen fixation, siderophore production, phosphate, potassium and zinc solubilization and phytohormone production (auxins, gibberellin, cytokinin). The role of endophytes in medicinal plants is especially significant as they have been found to act as alternative source of several bioactive metabolites. *Bacillus cereus, Gibberella moniliformis, Aspergillus terreus, A.flavus, A. niger, Enterobacter cancerogenus, E. cloaceae, E. asburiae* and *E. ludwiggi* are some of the endophytes isolated from medicinal plants and have been shown to produce secondary metabolites when cultured in vitro.

In the present study, *Pimenta dioica* (Allspice), Jamaican pepper, family: myrtaceae the plant with culinary and medicinal properties, will be used for isolation of endophytes. The oil extracted from Allspice have aroma of a combination of pepper, nutmeg, clove and cinnamon. The extracts of *Pimenta* leaves have been used as food fumigant to preserve freshness and sterility of meat and poultry products (Zabka et al. 2009, Martinez et al. 2011). Allspice is used for colds, dysmenorrhea (menstrual cramps), dyspepsia (upset stomach) and diabetes. Allspice berries are crushed and applied to bruises, sore joints and for myalgia (muscle ache). In Cuban medicine, Allspice along with other

herbal mixtures is used to relieve indigestion. In Europe, anecdotal uses of Allspice extract for dyspepsia and as purgative exist. Allspice is used to treat indigestion due to the abundance of the common polyphenol, Eugenol, which is known to stimulate digestive enzymes (Kamatou et al. 2012). Methanol extract of *P. dioica* is also used for menopause treatment (Doyle et al. 2009). Allspice berries are rich in polyphenols and kills breast cancer cells by autophagy and delays growth of triple negative breast cancer.

IMPORTANCE OF STUDY

The present study has been designed to determine the endophytic population from *P. dioica*. Several reports suggest the role of endophytes in imparting selective advantage to plants under nutrient limiting, biotic and abiotic stress conditions, and as a source of important secondary metabolites. In addition to providing a basic understanding of the diversity of microbial population from this species, it would also be interesting to study the role(s) of these endophytes in general plant growth and development.

BENEFITS BY ISOLATION OF THE ENDOPHYTES

Since endophytes occur within the plant tissues, they are apparently non-parasitic in nature. Over several years of evolution, they must have developed mutualistic association with the plant system. There is abundant literature on the role of endophytes in plant development under specific conditions like fixation of atmospheric nitrogen, production of phytohormones, defense mechanisms against abiotic and biotic stresses. There are reports showing the production of commercially important secondary metabolites by endophytes, they could, therefore, be used in the field of bioprospecting.

In addition to all the above points, the study of endophytes in *Pimenta dioica* will unravel the microbial diversity in this plant; some of which are also expected to have agriculturally important features.

FEASIBLITY OF ENDOPHYTES FROM AGRICULTURAL POINT OF VIEW

One of the motives behind this study is to identify some microorganisms that would have potential agronomic importance like potent biofertilizers (in the form of nitrogen fixation, increasing nutrient availability), biocontrol properties etc. These properties of endophytes are supported by literature.

OBJECTIVES

In view of the importance of the plant species and endophytes, the present study has been undertaken with the following objectives:

Following objectives could be formulated for this study:

1. Isolation of endophytes from different parts of *Pimenta dioica.*
2. Morphological and biochemical characterization of the isolated endophytes.
3. Identification of the isolated endophytes by rDNA sequencing.
4. Screening of endophytes for their effects on plant growth.

REVIEW OF LITERATURE

Endophytes are microbes which colonize living, internal tissues of plants without causing any harm to their host (Bacon and White, 2000). Endophytes have rich biodiversity and unexplored biosynthetic potential to produce an array of bioactive metabolites, especially in medicinal plants (Jasim et al. 2013). Endophytic microbes that have been exploited to produce therapeutically important products include vinblastine and vincristine, taxol, azadirachtin, podophyllotoxin, deoxypodophyllotoxin, camptothecin and hypericin (Zhang et al. 2006). Li et al. (2012) reported an endophytic actinobacteria, *Pseudonocardia*, from *Artimisia annua* which was found to enhance production of artemisinin. Sarang et al. (2017) reported an endophytic fungus, *Gibberella moniliformis*, from *Lawsonia inermis* which produces the orange dye, lawsone, independent of the plant. Pandey et al. (2016) reported two fungal endophytes, *Curvularia sps*. and *Choanephora infundibulifera*, from surface sterilized leaves of *Vinca rosea*, which were found to enhance production of vindoline content (229–403%) in host plant. Premjanu et al. (2016) have shown that *Aspergillus flavus* has maximum antifungal activity with an inhibitory zone of 26.22 mm against *Candida albicans* and 16.72 mm against *Malassezia pachydermis*. Ming et al. (2013) reported endophytic fungus *Trichoderma atroviride* from *Salvia miltiorrhiza* hairy roots that stimulated biosynthesis of tanshinone in the host plant by enhancing the expression of genes related to tanshinone biosynthesis. Hammerschmidt et al. (2012) reported fungal endophyte *Penicillium glabrum* isolated from *Punica granatum* (Pomegranate) produced two secondary metabolites, 3-methyldesmethoxyyangonin and 3-methylbisnoryangonin. Chitra et al. (2013) reported an endophytic fungus, *Colletotrichum gloeosporioides*, from *Piper nigrum* which enhances piperine production. In addition to these bioactive metabolties, endophytes have been reported to produce other commercially important products like industrial enzymes (amylase, tyrosinase and pectinase etc.), and biodegradable polymers like polyhydroxyalkanotaes (Pavithra et al. 2012; Das et al. 2017).

The role of endophytes in biotic and abiotic stress has also been well documented. Chemical pesticides are generally used to control plant pathogens, but their harmful effects on the ecosystem have forced considerable attention to the development of potential biocontrol agents and use of

endophytes as biopesticides could be a promising approach. Samuel et al. (2016) reported *Enterobacter cancerogenus, E.cloacae* and *E.asburiae* from roots of *Piper nigrum*, which had strong antagonism against *Phytophtora capsici*, a pathogen of foot rot and stem blight disease. Pleban et al. (1997 isolated an endophytic bacteria *Bacillus cereus* from mustard, and had biocontrol activity against *Rhizoctonia solani* by producing chitinase enzyme. Senthilkumar et al. (2009) isolated endophytic bacteria *Paenibacillus sps.* and *Bacillus sps.* from roots, nodule and stem of soyabean, and these isolates were having biocontrol activity against charcoal rot fungus *Rhizoctonia solanai*. Verma et al. (2009) isolated endophytic actinobacteria *Streptosporangium sp., Nocardia sps., Streptomyces sps*. from roots of *Azadriachtha indica*, which showed significant antagonistic activity against root pathogens, including *Pythium* and *Phytophthora sps.* There are few reports where endophytes protect plants from abiotic stresses as drought, temperature, high salinity etc. by enhancing level of certain secondary metabolites and proteins. Forchetti et al. (2007) reported endophytic bacteria *Achromobacter xiloxidans* and *Alcaligens sps*. From *Helianthus annus* under drought stressed conditions and these isolates enhanced production of phytohormones as abscisic acid and jasmonic acid. Khan et al. (2009) isolated several bacterial endophytes, *Enterobacter, Rahnella, Rhodanobacter, Pseudomonas, Stenotrophomonas, Xanthomonas* and *Phyllobacterium*, from *Ipomea batata*; some of these isolates were found to produce IAA.

The ability to produce siderophores, the iron chelating molecules, help in the uptake of iron from soils. Loaces et al. (2009) isolated some siderophore producing endophytes like *Sphingomonas, Pseudomonas, Burkholderia* and *Enterobacter*, from Rice. Taurian *et al.* (2010) reported some endophytic bacteria from Peanut, which helped in phosphate solubilisation. These endophytes were found to produce certain acids which convert insoluble phosphates to soluble phosphates. Nicholas et al. (2015) reported innoculation of endophyte *Pseudomonas* in *Pisum sativum* (pea), which solubilizes insoluble phosphate to soluble phosphate and increases biomass production. Ramesh Chand et al. (2017) reported the role of two potent bacterial strains *Fomitopsis meliae* and *Aspergillus tubingensis* in potassium solubilization.

The rhizosphere microorganisms have been widely reported to enhance phytoremediation (Lebeau *et al*. 2008; Gerhardt *et al*. 2009; Rajkumar *et al.* 2010). In addition, endophyte-assisted

phytoremediation has been documented as a promising technology for in situ remediation of contaminated soils. For the phytoremediation of organic contaminants and heavy metals, endophytes have been reported to produce various enzymes that degrade organic contaminants and reduce both the phytotoxicity and evapotranspiration of volatile contaminants. In comparison with rhizosphere microorganisms, endophytes interact more closely with their host plants and could more efficiently improve phytoremediation (Germaine *et al*. 2009; Weyens*et al*. 2009; 2010; Zhang *et al.* 2011). Chen *et al*. (2014) reported an endophyte, *Sphingomonas*, from *Sedum alfredi* with the potential to remediate zinc from soil and increases plant biomass. Yousaf *et al.* (2010) reported endophytic bacteria from (*Lolium multiflorum var. taurus*) and *Lotus corniculatus var. leo* which can degrade hydrocarbons. It was also found that many isolates were having alkane hydroxylase genes. Soleimani *et al*. (2010) demonstrated that endophytic fungi were helpful in the phytoremediation of an aged petroleum contaminated soil and that these fungi improved host plants' root and shoot biomass and created higher levels of water-soluble phenols and dehydrogenase activity in the soil. Guo *et al.* (2010) reported a cadmium hyper accumulator endophytic bacteria *Bacillus sps*. from *Solanum nigrum.* Nitrogen fixing endophytes like *Herbaspirillum* and *Acetobacter* have been reported from a number of plant species like wild rice (Adel et al. 2011) and sugarcance (Donget *et al*. 1994) respectively.

METHODOLOGY

Endophytes will be isolated from healthy and symptomless plants of *Pimenta dioica*, procured from regional station of NBPGR (National Bureau of Plant Genetic Resource), Thrisshur, Kerala; and maintained in the polyhouse of Dayalbagh Educational Institute, Agra.Various plant parts will be surface sterilized and inoculated on Nutrient Agar (NA) and Potato Dextrose (PDA) medium. Surface sterility will be checked by spreading 0.1 ml of the last rinsed water on PDA and NA plates. To distinguish endophytes from epiphytes, imprints of the various plant parts will be done on PDA and NA medium. (Verma et al. 2011). Petridishes will be sealed with parafilm and incubated at 26±2°C. Another method to isolate endophytes would involve grinding the sterilized plant material in PBS buffer, followed by centrifugation, serial dilution and spreading of 100 µl of supernatant on NA and PDA plates. Pure colonies would be obtained by sub-culturing and streaking of isolates and finally storing in -20°C as glycerol stocks.

Morphological and biochemical characterization of bacterial and fungal colonies: - Cellular morphology and motility of the isolated endophytes will be determined by light microscopy (Nikon E200, oil immersion, 100X objective). Also, plant tissue samples will be analysed under the microscope to determine the in-planta cellular localization of endophytes. For biochemical characterization, the endophytic isolates at exponential growth phase (OD ~ 0.2 - 0.4) will be used to perform the following tests:

1. Gram staining (Gram, 1884)
2. Indole-3-acetic acid production (Gordon and Weber, 1951)
3. Phosphate solubilization (Surange et al., 1997)
4. ACC deaminase production (Dworkin and Foster, 1958)
5. Siderophore production (Schwyn and Neilands, 1987).
6. Catalase activity (Graham and Parker, 1964)
7. Voges-Proskauer test (Dubey and Maheshwari, 2012)
8. Ammonia production (Cappuccino and Sherman, 1992)
9. Hydrogen cyanide production (Lorck, 1948)

Identification of the isolated endophytes

The bacterial and fungal endophytes isolated in this study will be identified by 16s rDNA and 18s rDNA sequencing, respectively. Genomic DNA from these endophytes would be used as template for amplification of 16S and 18S rDNA using their respective universal primers. This amplified DNA will be sequenced and identified by BLAST tool of NCBI.

Screening of the isolated endophytes for plant growth promoting traits

Beneficial and promising isolates identified on the basis of biochemical tests (mentioned above) will be used for pot experiments. Some cultivated plants will be inoculated with the endophytes in order to check its effect on plant growth and yield parameters. The information regarding properties and economic importance of the identified endophytes will be obtained from literature survey. Thereafter, experiments will be set up for each endophytic isolate. Accordingly it will be decided whether to do pot experiments or seed experiments. Appropriate controls will be maintained in each case. The effect of these endophytic isolates on plant growth parameters will be checked.

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